

APPLICATION FOR UNITED STATES LETTERS PATENT

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Title: VERSATILE HYDROPHILIC DYES

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SPECIFICATION

VERSATILE HYDROPHILIC DYES

This application is a continuation-in-part of application Serial No. 09/484,321, filed January 18, 2000.^A *now U.S. Patent No. 6,183,726*

Field of the Invention

5 This invention relates generally to compositions of cyanine dye bioconjugates with bioactive molecules for diagnosis and therapy, particularly, for visualization and detection of tumors.

Background of the Invention

10 Several dyes that absorb and emit light in the visible and near-infrared region of the electromagnetic spectrum are currently being used for various biomedical applications due to their biocompatibility, high molar absorptivity, and/or high fluorescence quantum yields. The high sensitivity of the optical modality in conjunction with dyes as contrast agents parallels that of nuclear medicine, and permits visualization of
15 organs and tissues without the undesirable effect of ionizing radiation.

 Cyanine dyes with intense absorption and emission in the near-infrared (NIR) region are particularly useful because biological tissues

are optically transparent in this region (B. C. Wilson, Optical properties of tissues. *Encyclopedia of Human Biology*, 1991, 5, 587-597). For example, indocyanine green, which absorbs and emits in the NIR region, has been used for monitoring cardiac output, hepatic functions, and liver blood flow (Y-L. He, et al., Measurement of blood volume using indocyanine green measured with pulse-spectrometry: Its reproducibility and reliability. *Critical Care Medicine*, 1998, 26(8), 1446-1451; J. Caesar, et al., The use of Indocyanine green in the measurement of hepatic blood flow and as a test of hepatic function. *Clin. Sci.* 1961, 21, 43-57), and its functionalized derivatives have been used to conjugate biomolecules for diagnostic purposes (R. B. Mujumdar, et al., Cyanine dye labeling reagents: Sulfoindocyanine succinimidyl esters. *Bioconjugate Chemistry*, 1993, 4(2), 105-111; U.S. Patent No. 5,453,505; WO 98/48846; WO 98/22146; WO 96/17628; WO 98/48838).

A major drawback in the use of cyanine dye derivatives is the potential for hepatobiliary toxicity resulting from the rapid clearance of these dyes by the liver (G. R. Cherrick, et al., Indocyanine green: Observations on its physical properties, plasma decay, and hepatic extraction. *J. Clinical Investigation*, 1960, 39, 592-600). This is associated with the tendency of cyanine dyes in solution to form aggregates, which could be taken up by Kupffer cells in the liver.

Various attempts to obviate this problem have not been very successful. Typically, hydrophilic peptides, polyethyleneglycol or

oligosaccharide conjugates have been used, but these resulted in long-circulating products, which are eventually still cleared by the liver. Another major difficulty with current cyanine and indocyanine dye systems is that they offer a limited scope in the ability to induce large changes in the absorption and emission properties of these dyes. Attempts have been made to incorporate various heteroatoms and cyclic moieties into the polyene chain of these dyes (L. Strekowski, et al., Substitution reactions of a nucleofugal group in hetamethine cyanine dyes. *J. Org. Chem.*, 1992, 57, 4578-4580; N. Narayanan and G. Patonay, A new method for the synthesis of heptamethine cyanine dyes: Synthesis of new near infrared fluorescent labels. *J. Org. Chem.*, 1995, 60, 2391-2395; U.S. Patent Nos. 5,732,104; 5,672,333; and 5,709,845), but the resulting dye systems do not show large differences in absorption and emission maxima, especially beyond 830 nm where photoacoustic diagnostic applications are very sensitive. They also possess a prominent hydrophobic core, which enhances liver uptake. Further, most cyanine dyes do not have the capacity to form starburst dendrimers, which are useful in biomedical applications.

For the purpose of tumor detection, many conventional dyes are useful for *in vitro* applications because of their highly toxic effect on both normal and abnormal tissues. Other dyes lack specificity for particular organs or tissues and, hence, must be attached to bioactive carriers such as proteins, peptides, carbohydrates, and the like to deliver

the dyes to specific regions in the body. Several studies on the use of near infrared dyes and dye-biomolecule conjugates have been published (G. Patonay and M. D. Antoine, Near-Infrared Fluorogenic Labels: New Approach to an Old Problem, *Analytical Chemistry*, 1991, 63:321A-327A and references therein; M. Brinkley, A Brief Survey of Methods for Preparing Protein Conjugates with Dyes, Haptens, and Cross-Linking Reagents, Perspectives in Bioconjugate Chemistry 1993, pp. 59-70, C. Meares (Ed), ACS Publication, Washington, DC; J. Slavik, Fluorescent Probes in Cellular and Molecular Biology, 1994, CRC Press, Inc.; U.S. Patent No. 5,453,505; WO 98/48846; WO 98/22146; WO 96/17628; WO 98/48838).

Of particular interest is the targeting of tumor cells with antibodies or other large protein carriers such as transferrin as delivery vehicles (A. Becker et al., "Transferrin Mediated Tumor Delivery of Contrast Media for Optical Imaging and Magnetic Resonance Imaging", Biomedical Optics meeting, January 23-29, 1999, San Jose, CA). Such an approach has been widely used in nuclear medicine applications. Its major advantage is the retention of a carrier's tissue specificity, since the molecular volume of the dye is substantially smaller than the carrier. However, this approach does have some serious limitations in that the diffusion of high molecular weight bioconjugates to tumor cells is highly unfavorable, and is further complicated by the net positive pressure in solid tumors (R. K. Jain, Barriers to Drug Delivery in Solid Tumors,

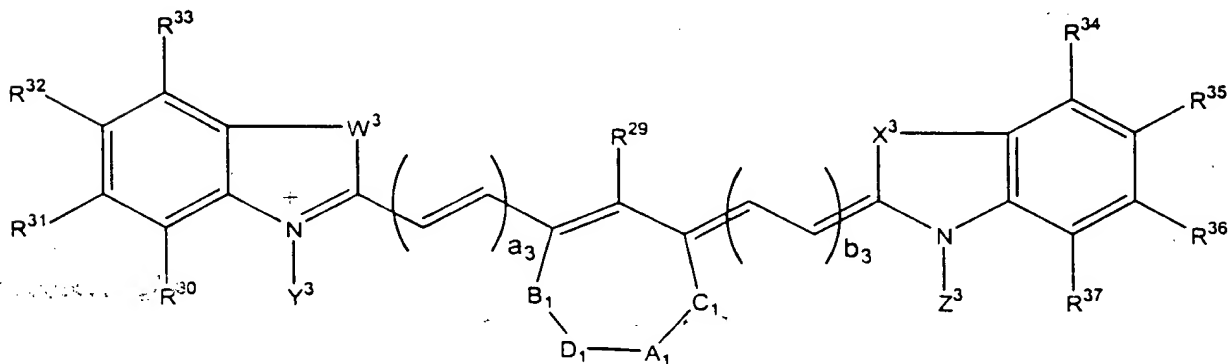
Scientific American 1994, 271:58-65. Furthermore, many dyes in general, and cyanine dyes in particular, tend to form aggregates in aqueous media that lead to fluorescence quenching.

Therefore, there is a need for dyes that could prevent dye aggregation in solution, that are predisposed to form dendrimers, that are capable of absorbing or emitting beyond 800 nm, that possess desirable photophysical properties, and that are endowed with tissue-specific targeting capability.

Summary of the Invention

The invention is directed to compositions, and methods of preparing the compositions, of low molecular weight biomolecule-dye conjugates to enhance tumor detection. The inventive compositions preserve the fluorescence efficiency of the dye molecules, do not aggregate in solution, form starburst dendrimers, are capable of absorbing or emitting light in the near infrared region (beyond 800 nm), and can be rendered tissue-specific.

In one embodiment, the inventive composition comprises cyanine dyes of general formula 1



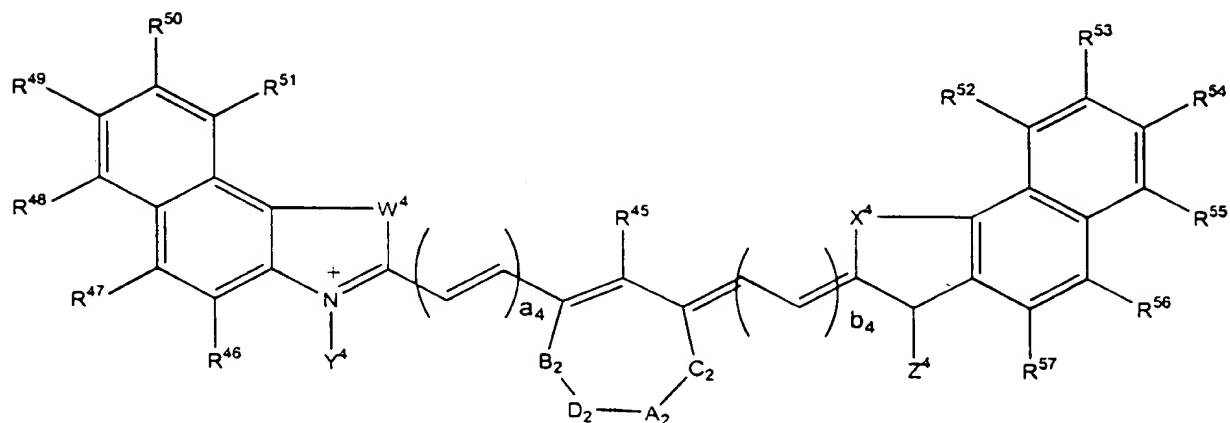
Formula 1

wherein W^3 and X^3 may be the same or different and are selected from the group consisting of $-CR^1R^2$, $-O-$, $-NR^3$, $-S-$, and $-Se$; Y^3 is selected from the group consisting of $-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$,
5 $-(CH_2)_a-N(R^3)-(CH_2)_b-CONH-Bm$, $(CH_2)_a-N(R^3)-(CH_2)_c-NHCO-Bm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-$
10 $(CH_2OCH_2)_d-NHCO-Bm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$;
 Z^3 is selected from the group consisting of $-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-(CH_2)_a-N(R^3)-(CH_2)_b-CONH-Dm$, $(CH_2)_a-N(R^3)-(CH_2)_c-NHCO-Dm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-N(R^3)-CH_2-$
15 $(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-NHCO-Dm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; A_1 is a single or a double bond; B_1 , C_1 , and D_1 may the
20 same or different and are selected from the group consisting of $-O-$, $-S-$, $-Se-$, $-P-$, $-CR^1R^2$, $-CR^1$, alkyl, NR^3 , and $-C=O$; A_1 , B_1 , C_1 , and D_1 may together form a 6- to 12-membered carbocyclic ring or a 6- to 12-membered heterocyclic ring optionally containing one or more oxygen,

nitrogen, or sulfur atom; a_3 and b_3 are independently from 0 to 5; R^1 to R^4 , and R^{29} to R^{37} are independently selected from the group consisting of hydrogen, C_1 - C_{10} alkyl, C_5 - C_{20} aryl, C_1 - C_{10} alkoxy, C_1 - C_{10}

- 5 C_{10} polyalkoxyalkyl, C_1 - C_{20} polyhydroxyalkyl, C_5 - C_{20} polyhydroxyaryl, C_1 -
 C_{10} aminoalkyl, cyano, nitro, halogen, saccharide, peptide,
 $-\text{CH}_2(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{OH}$, $-(\text{CH}_2)_a-\text{CO}_2\text{H}$, $-(\text{CH}_2)_a-\text{CONH-Bm}$, $-\text{CH}_2-$
 $(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{CONH-Bm}$, $-(\text{CH}_2)_a-\text{NHCO-Bm}$, $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-$
 NHCO-Bm , $-(\text{CH}_2)_a-\text{OH}$ and $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CO}_2\text{H}$; Bm and Dm are
independently selected from the group consisting of a bioactive
10 peptide, a protein, a cell, an antibody, an antibody fragment, a
saccharide, a glycopeptide, a peptidomimetic, a drug, a drug mimic, a
hormone, a metal chelating agent, a radioactive or nonradioactive
metal complex, and an echogenic agent; a and c are independently
from 1 to 20; and b and d are independently from 1 to 100.

- 15 In a second embodiment, the inventive composition
comprises cyanine dyes of general formula 2

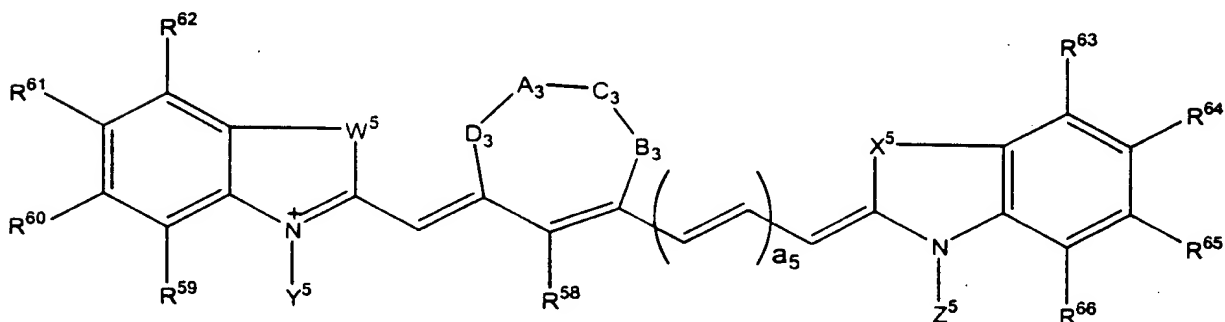


Formula 2

wherein W^4 and X^4 may be the same or different and are selected from the group consisting of $-CR^1R^2$, $-O-$, $-NR^3$, $-S-$, and $-Se$; Y^4 is selected from the group consisting of $-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$,
5 $-(CH_2)_a-N(R^3)-(CH_2)_b-CONH-Bm$, $(CH_2)_a-N(R^3)-(CH_2)_c-NHCO-Bm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-$
10 $(CH_2OCH_2)_d-NHCO-Bm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$;
 Z^4 is selected from the group consisting of $-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-(CH_2)_a-N(R^3)-(CH_2)_b-CONH-Dm$, $(CH_2)_a-N(R^3)-(CH_2)_c-NHCO-Dm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-N(R^3)-CH_2-$
15 $(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-NHCO-Dm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; A_2 is a single or a double bond; B_2 , C_2 , and D_2 may be the same or different and are selected from the group consisting of $-O-$,
20 $-S-$, $-Se-$, $-P-$, $-CR^1R^2$, $-CR^1$, alkyl, NR^3 , and $-C=O$; A_2 , B_2 , C_2 , and D_2 may together form a 6- to 12-membered carbocyclic ring or a 6- to

12-membered heterocyclic ring optionally containing one or more oxygen, nitrogen, or sulfur atom; a_4 and b_4 independently vary from 0 to 5; R^1 to R^4 , and R^{45} to R^{57} are independently selected from the group consisting of hydrogen, C_1 - C_{10} alkyl, C_5 - C_{20} aryl, C_1 - C_{10} alkoxy, C_1 - C_{10} polyalkoxyalkyl, C_1 - C_{20} polyhydroxyalkyl, C_5 - C_{20} polyhydroxyaryl, C_1 - C_{10} aminoalkyl, cyano, nitro, halogen, saccharide, peptide, $-\text{CH}_2(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{OH}$, $-(\text{CH}_2)_a-\text{CO}_2\text{H}$, $-(\text{CH}_2)_a-\text{CONH-Bm}$, $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{CONH-Bm}$, $-(\text{CH}_2)_a-\text{NHCO-Bm}$, $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{NHCO-Bm}$, $-(\text{CH}_2)_a-\text{OH}$ and $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CO}_2\text{H}$; Bm and Dm are independently selected from the group consisting of a bioactive peptide, a protein, a cell, an antibody, an antibody fragment, a saccharide, a glycopeptide, a peptidomimetic, a drug, a drug mimic, a hormone, a metal chelating agent, a radioactive or nonradioactive metal complex, and an echogenic agent; a and c are independently from 1 to 20; and b and d are independently from 1 to 100.

In a third embodiment, the inventive composition comprises cyanine dyes of general formula 3

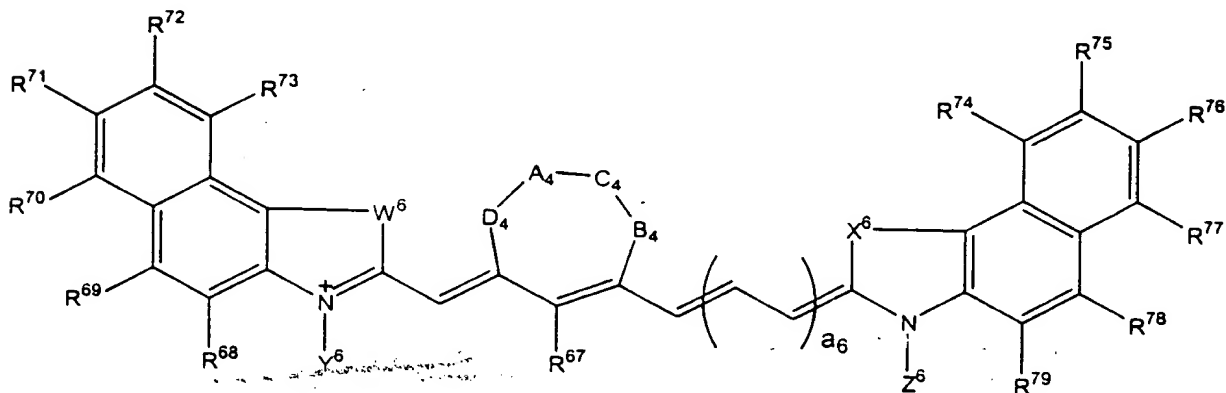


Formula 3

wherein W^5 and X^5 may be the same or different and are selected from the group consisting of $-CR^1R^2$, $-O-$, $-NR^3$, $-S-$, and $-Se$; Y^5 is selected from the group consisting of $-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$,
5 $-(CH_2)_a-N(R^3)-(CH_2)_b-CONH-Bm$, $(CH_2)_a-N(R^3)-(CH_2)_c-NHCO-Bm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-$
10 $(CH_2OCH_2)_d-NHCO-Bm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$;
 Z^5 is selected from the group consisting of $-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-(CH_2)_a-N(R^3)-(CH_2)_b-CONH-Dm$, $(CH_2)_a-N(R^3)-(CH_2)_c-NHCO-Dm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-N(R^3)-CH_2-$
15 $(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-NHCO-Dm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; A_3 is a single or a double bond; B_3 , C_3 , and D_3 may be the same or different and are selected from the group consisting of $-O-$,
20 $-S-$, $-Se-$, $-P-$, $-CR^1R^2$, $-CR^1$, alkyl, NR^3 , and $-C=O$; A_3 , B_3 , C_3 , and D_3 may together form a 6- to 12-membered carbocyclic ring or a 6- to 12-membered heterocyclic ring.

- 12-membered heterocyclic ring optionally containing one or more oxygen, nitrogen, or sulfur atom; a_5 is independently from 0 to 5; R^1 to R^4 , and R^{58} to R^{66} are independently selected from the group consisting of hydrogen, C_1 - C_{10} alkyl, C_5 - C_{20} aryl, C_1 - C_{10} alkoxy, C_1 - C_{10}
- 5 polyalkoxyalkyl, C_1 - C_{20} polyhydroxyalkyl, C_5 - C_{20} polyhydroxyaryl, C_1 - C_{10} aminoalkyl, cyano, nitro, halogen, saccharide, peptide, $-\text{CH}_2(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{OH}$, $-(\text{CH}_2)_a-\text{CO}_2\text{H}$, $-(\text{CH}_2)_a-\text{CONH-Bm}$, $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{CONH-Bm}$, $-(\text{CH}_2)_a-\text{NHCO-Bm}$, $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{NHCO-Bm}$, $-(\text{CH}_2)_a-\text{OH}$ and $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CO}_2\text{H}$; Bm and Dm are
- 10 independently selected from the group consisting of a bioactive peptide, a protein, a cell, an antibody, an antibody fragment, a saccharide, a glycopeptide, a peptidomimetic, a drug, a drug mimic, a hormone, a metal chelating agent, a radioactive or nonradioactive metal complex, and an echogenic agent; a and c are independently
- 15 from 1 to 20; and b and d are independently from 1 to 100.

In a fourth embodiment, inventive composition comprises cyanine dyes of general formula 4



Formula 4

- wherein W^6 and X^6 may be the same or different and are selected from the group consisting of $-CR^1R^2$, $-O-$, $-NR^3$, $-S-$, and $-Se$; Y^6 is selected from the group consisting of $-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$,
- 5 $-(CH_2)_a-N(R^3)-(CH_2)_b-CONH-Bm$, $(CH_2)_a-N(R^3)-(CH_2)_c-NHCO-Bm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-$
- 10 $(CH_2OCH_2)_d-NHCO-Bm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; Z^6 is selected from the group consisting of $-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-(CH_2)_a-N(R^3)-(CH_2)_b-CONH-Dm$, $(CH_2)_a-N(R^3)-(CH_2)_c-NHCO-Dm$, $-(CH_2)_a-N(R^3)-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-N(R^3)-CH_2-$
- 15 $(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-N(R^3)-CH_2-(CH_2OCH_2)_d-NHCO-Dm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; A_4 is a single or a double bond; B_4 , C_4 , and D_4 may the
- 20 same or different and are selected from the group consisting of $-O-$, $-S-$, $-Se-$, $-P-$, $-CR^1R^2$, $-CR^1$, alkyl, NR^3 , and $-C=O$; A_4 , B_4 , C_4 , and D_4 may together form a 6- to 12-membered carbocyclic ring or a 6- to

12-membered heterocyclic ring optionally containing one or more oxygen, nitrogen, or sulfur atom; a_6 is independently from 0 to 5; R^1 to R^4 , and R^{67} to R^{79} are independently selected from the group consisting of hydrogen, C_1 - C_{10} alkyl, C_5 - C_{20} aryl, C_1 - C_{10} alkoxy, C_1 - C_{10} polyalkoxyalkyl, C_1 - C_{20} polyhydroxyalkyl, C_5 - C_{20} polyhydroxyaryl, C_1 - C_{10} aminoalkyl, cyano, nitro, halogen, saccharide, peptide, $-CH_2(CH_2OCH_2)_b-CH_2-OH$, $-(CH_2)_a-CO_2H$, $-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$, $-(CH_2)_a-OH$ or $-CH_2-(CH_2OCH_2)_b-CO_2H$; Bm and Dm are independently selected from the group consisting of a bioactive peptide, a protein, a cell, an antibody, an antibody fragment, a saccharide, a glycopeptide, a peptidomimetic, a drug, a drug mimic, a hormone, a metal chelating agent, a radioactive and nonradioactive metal complex, and an echogenic agent; a and c are independently from 1 to 20; and b and d are independently from 1 to 100.

The invention will be further appreciated in light of the following figures, detailed description, and examples.

Brief Description of the Figures

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

FIG. 1 shows the reaction pathway for the synthesis of bis-carboxylic acid cyanine dyes.

FIG. 2 shows the reaction pathway for the synthesis of tetracarboxylic acid cyanine dyes.

5 FIG. 3 shows the reaction pathway for the synthesis of polyhydroxycarboxylic acid dyes.

FIG. 4 shows the reaction pathway for the synthesis of non-aggregating cyanine dyes.

10 FIG. 5 shows the reaction pathway for the synthesis of long wavelength absorbing dyes.

FIG. 6 shows the reaction pathway for the synthesis of cyanine dye bioconjugates.

FIGS. 7A-F represent images at 2 minutes and 30 minutes post injection of indocyanine green (ICG) into rats with various tumors.

15 FIGS. 8A-B show a comparison of the uptake of ICG (FIG. 8A) and Cytate 1 (FIG. 8B) in rats with the pancreatic acinar carcinoma (CA20948).

FIGS. 9A-B show images of rats with the pancreatic acinar carcinoma (CA20948) 45 minutes (FIG. 9A) and 27 hours
20 (FIG. 9B) post injection of Cytate 1.

FIGS. 10 is an image of individual organs taken from a rat with pancreatic acinar carcinoma (CA20948) about 24 hours after injection with Cytate 1.

FIG. 11 is an image of bombesinate in an AR42-J tumor-bearing rat 22 hours after injection.

FIG. 12 is the clearance profile of Cytate 1 from the blood of a normal rat.

5 FIG. 13 is the clearance profile of Cytate 1 from the blood of a pancreatic tumor-bearing rat.

FIG. 14 is the clearance profile of Cytate 2 from the blood of a normal rat.

10 FIG. 15 is the clearance profile of Cytate 2 from the blood of a pancreatic tumor-bearing rat.

FIG. 16 is the clearance profile of Cytate 4 from the blood of a normal rat.

Detailed Description of the Invention

The novel compositions of the present invention

15 comprising dyes of formulas 1 to 4 offer significant advantages over those currently described in the art. These inventive dyes form starburst dendrimers which prevent aggregation in solution by preventing intramolecular and intermolecular ordered hydrophobic interactions, and have multiple attachment sites proximal to the dye

20 chromophore for ease of forming bioactive molecules. The presence of rigid and extended chromophore backbone enhances their fluorescence quantum yield and extends their maximum absorption beyond 800 nm. Conjugation of biomolecules to these dyes is readily achievable.

The inventive bioconjugates of the present invention also exploit the symmetric nature of the cyanine and indocyanine dye structures by incorporating one to ten receptor targeting groups in close proximity to each other, such that the receptor binding can be greatly enhanced due to a cooperative effect. Accordingly, several cyanine dyes containing one or more targeting domains have been prepared and tested *in vivo* for biological activity.

The inventive dye-bioconjugates of formulas 1 to 4 are useful for various biomedical applications. These include, but are not limited to, tomographic imaging of organs, monitoring of organ functions, coronary angiography, fluorescence endoscopy, detection, imaging, and therapy of tumors, laser assisted guided surgery, photoacoustic methods, and sonofluorescent methods.

Specific embodiments to accomplish some of the aforementioned biomedical applications are given below. The novel dyes of the present invention are prepared according the methods well known in the art and are illustrated in FIGS. 1-5.

FIG. 1 illustrates the synthetic scheme for bis-carboxylic acid cyanine dyes, where $A = CH_2$ or CH_2OCH_2 ; $R = COOH$; $R' = COOH$, $NHFMoc$; CO_2t-Bu ; SO_3^- ; $R_1 = R_2 = H$ (Formula 1) or $R_1, R_2 =$ fused phenyl (Formula 2).

FIG. 2 illustrates the synthetic scheme for tetracarboxylic acid cyanine dyes, where $A = CH_2$ or CH_2OCH_2 ; $R_1 = R_2 = H$ (Formula 1) or $R_1, R_2 =$ fused phenyl (Formula 2).

FIG. 3 illustrates the synthetic scheme for polyhydroxy-carboxylic acid cyanine dyes.

FIG. 4 illustrates the synthetic scheme for non-aggregating cyanine dyes.

FIG. 5 illustrates the synthetic scheme for long wavelength-absorbing "tunable" cyanine dyes.

In one embodiment, the inventive bioconjugates have the Formula 1, wherein W^3 and X^3 may be the same or different and are selected from the group consisting of $-C(CH_3)_2$, $-C((CH_2)_aOH)CH_3$, $-C((CH_2)_aOH)_2$, $-C((CH_2)_aCO_2H)CH_3$, $-C((CH_2)_aCO_2H)_2$, $-C((CH_2)_aNH_2)CH_3$, $-C((CH_2)_aNH_2)_2$, $-C((CH_2)_aNR^3R^4)_2$, $-NR^3$, and $-S-$; Y^3 is selected from the group consisting of $-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; Z^3 is selected from the group consisting of $-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; A_1 is a single or a double bond; B_1 , C_1 , and D_1 are independently selected from the group consisting of $-O-$, $-S-$, NR^3 , $(CH_2)_a-CR^1R^2$, and $-CR^1$; A_1 , B_1 , C_1 , and D_1 may together form a 6- to 10-membered carbocyclic ring or

a 6- to 10-membered heterocyclic ring optionally containing one or more oxygen, nitrogen, or sulfur atom; a_3 and b_3 are independently from 0 to 3; R^1 to R^4 , and R^{29} to R^{37} are independently selected from the group consisting of hydrogen, C_1 - C_{10} alkyl, C_5 - C_{12} aryl, C_1 - C_{10} alkoxy, C_1 - C_{10} polyhydroxyalkyl, C_5 - C_{12} polyhydroxyaryl, C_1 - C_{10} aminoalkyl, mono- or oligosaccharide, peptide with 2 to 30 amino acid units, $-\text{CH}_2(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{OH}$, $-(\text{CH}_2)_a-\text{CO}_2\text{H}$, $-(\text{CH}_2)_a-\text{CONH-Bm}$, $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{CONH-Bm}$, $-(\text{CH}_2)_a-\text{NHCO-Bm}$, $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{NHCO-Bm}$, $-(\text{CH}_2)_a-\text{OH}$ and $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CO}_2\text{H}$; Bm and Dm are independently selected from the group consisting of a bioactive peptide containing 2 to 30 amino acid units, an antibody, a mono- or oligosaccharide, a glycopeptide, a metal chelating agent, a radioactive or nonradioactive metal complex, and an echogenic agent; a and c are independently from 1 to 10; and b and d are independently from 1 to 30.

In a second embodiment, the inventive bioconjugates have the general Formula 2, wherein W^4 and X^4 may be the same or different and are selected from the group consisting of $-\text{C}(\text{CH}_3)_2$, $-\text{C}((\text{CH}_2)_a\text{OH})\text{CH}_3$, $-\text{C}((\text{CH}_2)_a\text{OH})_2$, $-\text{C}((\text{CH}_2)_a\text{CO}_2\text{H})\text{CH}_3$, $-\text{C}((\text{CH}_2)_a\text{CO}_2\text{H})_2$, $-\text{C}((\text{CH}_2)_a\text{NH}_2)\text{CH}_3$, $-\text{C}((\text{CH}_2)_a\text{NH}_2)_2$, $-\text{C}((\text{CH}_2)_a\text{NR}^3\text{R}^4)_2$, $-\text{NR}^3$, and $-\text{S}-$; Y^4 is selected from the group consisting of $-(\text{CH}_2)_a-\text{CONH-Bm}$, $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{CONH-Bm}$, $-(\text{CH}_2)_a-\text{NHCO-Bm}$, $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{NHCO-Bm}$, $-(\text{CH}_2)_a-\text{NR}^3\text{R}^4$, and

-CH₂(CH₂OCH₂)_b-CH₂NR³R⁴; Z⁴ is selected from the group consisting of
-(CH₂)_a-CONH-Dm, -CH₂-(CH₂OCH₂)_b-CH₂-CONH-Dm, -(CH₂)_a-NHCO-
Dm, -CH₂-(CH₂OCH₂)_b-CH₂-NHCO-Dm, -(CH₂)_a-NR³R⁴, and
-CH₂(CH₂OCH₂)_b-CH₂NR³R⁴; A₂ is a single or a double bond; B₂, C₂, and
5 D₂ are independently selected from the group consisting of
-O-, -S-, NR³, (CH₂)_a-CR¹R², and -CR¹; A₂, B₂, C₂, and D₂ may together
form a 6- to 10-membered carbocyclic ring or a 6- to 10-membered
heterocyclic ring optionally containing one or more oxygen, nitrogen, or
sulfur atom; a₄ and b₄ are independently from 0 to 3; R¹ to R⁴, and R⁴⁵
10 to R⁵⁷ are independently selected from the group consisting of
hydrogen, C₁-C₁₀ alkyl, C₅-C₁₂ aryl, C₁-C₁₀ alkoxy, C₁-C₁₀
polyhydroxyalkyl, C₅-C₁₂ polyhydroxyaryl, C₁-C₁₀ aminoalkyl, mono- or
oligosaccharide, peptide with 2 to 30 amino acid units,
-CH₂(CH₂OCH₂)_b-CH₂-OH, -(CH₂)_a-CO₂H,
15 -(CH₂)_a-CONH-Bm, -CH₂-(CH₂OCH₂)_b-CH₂-CONH-Bm, -(CH₂)_a-NHCO-Bm,
-CH₂-(CH₂OCH₂)_b-CH₂-NHCO-Bm, -(CH₂)_a-OH and -CH₂-(CH₂OCH₂)_b-
CO₂H; Bm and Dm are independently selected from the group
consisting of a bioactive peptide containing 2 to 30 amino acid units,
an antibody, a mono- or oligosaccharide, a glycopeptide, a metal
20 chelating agent, a radioactive or nonradioactive metal complex, and an
echogenic agent; a and c are independently from 1 to 10; and b and d
are independently from 1 to 30.

In a third embodiment, the inventive bioconjugates have the general Formula 3, wherein W^5 and X^5 may be the same or different and are selected from the group consisting of $-C(CH_3)_2$, $-C((CH_2)_aOH)CH_3$, $-C((CH_2)_aOH)_2$, $-C((CH_2)_aCO_2H)CH_3$, $-C((CH_2)_aCO_2H)_2$, $-C((CH_2)_aNH_2)CH_3$, $-C((CH_2)_aNH_2)_2$, $-C((CH_2)_aNR^3R^4)_2$, $-NR^3$, and $-S-$; Y^5 is selected from the group consisting of $-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-NHCO-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Bm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; Z^5 is selected from the group consisting of $-(CH_2)_a-CONH-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Dm$, $-(CH_2)_a-NHCO-Dm$, $-CH_2-(CH_2OCH_2)_b-CH_2-NHCO-Dm$, $-(CH_2)_a-NR^3R^4$, and $-CH_2(CH_2OCH_2)_b-CH_2NR^3R^4$; A_3 is a single or a double bond; B_3 , C_3 , and D_3 are independently selected from the group consisting of $-O-$, $-S-$, NR^3 , $(CH_2)_a-CR^1R^2$, and $-CR^1$; A_3 , B_3 , C_3 , and D_3 may together form a 6- to 10-membered carbocyclic ring or a 6- to 10-membered heterocyclic ring optionally containing one or more oxygen, nitrogen, or sulfur atom; a_5 is from 0 to 3; R^1 to R^4 , and R^{58} to R^{66} are independently selected from the group consisting of hydrogen, C_1 - C_{10} alkyl, C_5 - C_{12} aryl, C_1 - C_{10} alkoxy, C_1 - C_{10} polyhydroxyalkyl, C_5 - C_{12} polyhydroxyaryl, C_1 - C_{10} aminoalkyl, mono- or oligosaccharide, peptide with 2 to 30 amino acid units, $-CH_2(CH_2OCH_2)_b-CH_2-OH$, $-(CH_2)_a-CO_2H$, $-(CH_2)_a-CONH-Bm$, $-CH_2-(CH_2OCH_2)_b-CH_2-CONH-Bm$, $-(CH_2)_a-NHCO-Bm$,

-CH₂-(CH₂OCH₂)_b-CH₂-NHCO-Bm, -(CH₂)_a-OH and -CH₂-(CH₂OCH₂)_b-CO₂H; Bm and Dm are independently selected from the group consisting of a bioactive peptide containing 2 to 30 amino acid units, an antibody, a mono- or oligosaccharide, a glycopeptide, a metal

5 chelating agent, a radioactive or nonradioactive metal complex, and an echogenic agent; a and c are independently from 1 to 10; and b and d are independently from 1 to 30.

In a fourth embodiment, the inventive bioconjugates have the general Formula 4, wherein W⁶ and X⁶ may be the same or

10 different and are selected from the group consisting of -C(CH₃)₂, -C((CH₂)_aOH)CH₃, -C((CH₂)_aOH)₂, -C((CH₂)_aCO₂H)CH₃, -C((CH₂)_aCO₂H)₂, -C((CH₂)_aNH₂)CH₃, -C((CH₂)_aNH₂)₂, C((CH₂)_aNR³R⁴)₂, -NR³, and -S-; Y⁶ is selected from the group consisting of -(CH₂)_a-CONH-Bm, -CH₂-(CH₂OCH₂)_b-CH₂-CONH-Bm, -(CH₂)_a-NHCO-Bm,

15 -CH₂-(CH₂OCH₂)_b-CH₂-NHCO-Bm, -(CH₂)_a-NR³R⁴, and -CH₂-(CH₂OCH₂)_b-CH₂NR³R⁴; Z⁶ is selected from the group consisting of -(CH₂)_a-CONH-Dm, -CH₂-(CH₂OCH₂)_b-CH₂-CONH-Dm, -(CH₂)_a-NHCO-Dm, -CH₂-(CH₂OCH₂)_b-CH₂-NHCO-Dm, -(CH₂)_a-NR³R⁴, and -CH₂-(CH₂OCH₂)_b-CH₂NR³R⁴; A₄ is a single or a double bond; B₄, C₄, and

20 D₄ are independently selected from the group consisting of -O-, -S-, NR³, (CH₂)_a-CR¹R², and -CR¹; A₄, B₄, C₄, and D₄ may together form a 6- to 10-membered carbocyclic ring or a 6- to 10-membered heterocyclic ring optionally containing one or more oxygen, nitrogen, or sulfur

atom; a_6 is from 0 to 3; R^1 to R^4 , and R^{67} to R^{79} are independently selected from the group consisting of hydrogen, C_1 - C_{10} alkyl, C_5 - C_{12} aryl, C_1 - C_{10} alkoxy, C_1 - C_{10} polyhydroxyalkyl, C_5 - C_{12} polyhydroxyaryl, C_1 - C_{10} aminoalkyl, mono- or oligosaccharide, peptide with 2 to 30
5 amino acid units, $-\text{CH}_2(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{OH}$, $-(\text{CH}_2)_a-\text{CO}_2\text{H}$, $-(\text{CH}_2)_a-\text{CONH-Bm}$, $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{CONH-Bm}$, $-(\text{CH}_2)_a-\text{NHCO-Bm}$, $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CH}_2-\text{NHCO-Bm}$, $-(\text{CH}_2)_a-\text{OH}$ and $-\text{CH}_2-(\text{CH}_2\text{OCH}_2)_b-\text{CO}_2\text{H}$; Bm and Dm are independently selected from the group consisting of a bioactive peptide containing 2 to 30 amino acid units,
10 an antibody, a mono- or oligosaccharide, a glycopeptide, a metal chelating agent, a radioactive or nonradioactive metal complex, and an echogenic agent; a and c are independently from 1 to 10; and b and d are independently from 1 to 30.

This invention is also related to the method of conjugating
15 the inventive dyes to peptides or biomolecules by solid phase or solution synthesis methods. FIG. 6 illustrates the synthetic scheme for bioconjugates incorporating the cyanine dyes of FIGS. 1-5, using automated peptide synthesis in a solid support, is shown in FIG. 6, where $A = \text{CH}_2$ or CH_2OCH_2 ; $R_1 = R_2 = \text{H}$ (Formula 1) or $R_1, R_2 =$
20 fused phenyl (Formula 2); AA = amino acids; R = CONH peptide; $R' = \text{R}$ (bis conjugate) or COOH (mono conjugate); $\textcircled{P} =$ solid support; $\textcircled{P}' =$ presence or absence depends on R' definition.

This invention is also related to the method of preventing fluorescence quenching. It is known that cyanine dyes generally form aggregates in aqueous media, leading to fluorescence quenching.

Where the presence of a hydrophobic core in the dyes leads to
5 fluorescence quenching, the addition of a biocompatible organic solvent, such as 1-50% dimethylsulfoxide (DMSO) for example, restored fluorescence by preventing aggregation and allowed *in vivo* organ visualization.

The inventive dye-biomolecule conjugates are used for
10 optical tomographic, endoscopic, photoacoustic and sonofluorescent applications for the detection and treatment of tumors and other abnormalities.

Dye-biomolecule conjugates are also used for localized therapy. This may be accomplished by attaching a porphyrin or other
15 photodynamic therapy agent to a bioconjugate, shining light of an appropriate wavelength to activate the agent, and detecting and/or treating the abnormality.

The inventive conjugates can also be used for the detection of the presence of tumors and other abnormalities by
20 monitoring the blood clearance profile of the conjugates, for laser assisted guided surgery for the detection of small micrometastases of, e.g., somatostatin subtype 2 (SST-2) positive tumors, upon

laparoscopy, and for diagnosis of atherosclerotic plaques and blood clots.

The compositions of the invention can be formulated into diagnostic and therapeutic compositions for enteral or parenteral administration. These compositions contain an effective amount of the dye along with conventional pharmaceutical carriers and excipients appropriate for the type of administration contemplated. For example, parenteral formulations advantageously contain the inventive agent in a sterile aqueous solution or suspension. Parenteral compositions may be injected directly or mixed with a large volume parenteral composition for systemic administration. Such solutions also may contain pharmaceutically acceptable buffers and, optionally, electrolytes such as sodium chloride.

Formulations for enteral administration may vary widely, as is well known in the art. In general, such formulations are liquids, which include an effective amount of the inventive agent in aqueous solution or suspension. Such enteral compositions may optionally include buffers, surfactants, thixotropic agents, and the like. Compositions for oral administration may also contain flavoring agents and other ingredients for enhancing their organoleptic qualities.

The diagnostic compositions are administered in doses effective to achieve the desired enhancement. Such doses may vary widely, depending upon the particular dye employed, the organs or

tissues to be imaged, the imaging equipment being used, and the like. The diagnostic compositions of the invention are used in the conventional manner. The compositions may be administered to a patient, typically a warm-blooded animal, either systemically or locally to the organ or tissue to be imaged, and the patient is then subjected to the imaging procedure.

The inventive compositions and methods represent an important approach to the synthesis and use of novel cyanine and indocyanine dyes with a variety of photophysical and chemical properties. The combination also represents an important approach to the use of small molecular targeting groups to image tumors by optical methods. The invention is further detailed in the following Examples, which are offered by way of illustration and are not intended to limit the scope of the invention in any manner.

EXAMPLE 1

Synthesis of Bis(ethylcarboxymethyl)indocyanine Dye (FIG. 1, $R_1, R_2 =$ fused phenyl; $A = CH_2$, $n = 1$ and $R = R' = CO_2H$)

A mixture of 1,1,2-trimethyl-[1H]-benz[e]indole (9.1 g, 43.58 mmoles) and 3-bromopropanoic acid (10.0 g, 65.37 mmoles) in 1,2-dichlorobenzene (40 mL) was heated at 110°C for 12 hours. The solution was cooled to room temperature and the red residue obtained was filtered and washed with acetonitrile:diethyl ether (1:1) mixture. The solid obtained was dried under vacuum to give 10 g (64%) of light

brown powder. A portion of this solid (6.0 g; 16.56 mmoles),
glutaconaldehyde dianil monohydrochloride (2.36 g, 8.28 mmoles),
and sodium acetate trihydrate (2.93 g, 21.53 mmoles) in ethanol (150
5 mL of 2 N aqueous HCl was added to the residue. The mixture was
centrifuged and the supernatant was decanted. This procedure was
repeated until the supernatant became nearly colorless. About 5 mL of
water:acetonitrile (3:2) mixture was added to the solid residue and
lyophilized to obtain 2 g of dark green flakes. The purity of the
10 compound was established with ^1H -NMR and liquid chromatography-
mass spectroscopy (LC-MS).

EXAMPLE 2

15 Synthesis of Bis(pentylcarboxymethyl)indocyanine Dye (FIG. 1, R_1 , R_2
= fused phenyl; $A = \text{CH}_2$, $n = 4$ and $R = R' = \text{CO}_2\text{H}$)

A mixture of 1,1,2-trimethyl-[1H]-benz[e]indole (20 g,
95.6 mmoles) and 6-bromohexanoic acid (28.1 g, 144.1 mmoles) in
1,2-dichlorobenzene (250 mL) was heated at 110°C for 12 hours.
20 The green solution was cooled to room temperature and the brown
solid precipitate formed was collected by filtration. After washing the
solid with 1,2-dichlorobenzene and diethyl ether, the brown powder
obtained (24 g, 64%) was dried under vacuum at room temperature.
A portion of this solid (4.0 g; 9.8 mmoles), glutaconaldehyde dianil
25 monohydrochloride (1.4 g, 5 mmoles) and sodium acetate trihydrate

(1.8 g, 12.9 mmoles) in ethanol (80 mL) were refluxed for 1 hour.

After evaporating the solvent, 20 mL of 2 N aqueous HCl was added to the residue. The mixture was centrifuged and the supernatant was decanted. This procedure was repeated until the supernatant became
5 nearly colorless. About 5 mL of water:acetonitrile (3:2) mixture was added to the solid residue and lyophilized to obtain about 2 g of dark green flakes. The purity of the compound was established with $^1\text{H-NMR}$ and LC-MS.

10

EXAMPLE 3

Synthesis of Bisethylcarboxymethylindocyanine Dye (FIG. 1, $R_1 = R_2 = \text{H}$; $A = \text{CH}_2$, $n = 1$ and $R = R' = \text{CO}_2\text{H}$)

15

This compound was prepared as described in Example 1 except that 1,1,2-trimethylindole was used as the starting material.

EXAMPLE 4

20

Synthesis of Bis(hexaethyleneglycolcarboxymethyl)indocyanine Dye (FIG. 1, $R_1 = R_2 = \text{fused phenyl}$; $A = \text{CH}_2\text{OCH}_2$, $n = 6$ and $R = R' = \text{CO}_2\text{H}$)

25

This compound was prepared as described in Example 1 except that ω -bromohexaoxyethyleneglycolpropionic acid was used in place of bromopropanoic acid and the reaction was carried out in 1,2-dimethoxypropane.

EXAMPLE 5

Synthesis of Bisethylcarboxymethylindocyanine Dye (FIG. 2, $R_1 = R_2 =$ fused phenyl; $A = CH_2$, and $n = 0$)

5 A solution of 50 ml of dimethylformamide and benzyl
bromoacetate (16.0 g, 70 mmol) was stirred in a 100 ml three-neck
flask. Solid potassium bicarbonate (7.8 g, 78 mmol) was added. The
flask was purged with argon and cooled to 0°C with an ice bath. To
the stirring mixture was added dropwise a solution of ethanolamine
10 (1.9 g, 31 mmol) and 4 ml of dimethylformamide over 5 minutes.
After the addition was complete the mixture was stirred for 1 hour at
0°C. The ice bath was removed and the mixture was stirred at room
temperature overnight. The reaction mixture was partitioned between
100 ml of methylene chloride and 100 ml of saturated sodium
15 bicarbonate solution. The layers were separated and the methylene
chloride layer was again washed with 100 ml of saturated sodium
bicarbonate solution. The combined aqueous layers were extracted
twice with 25 ml of methylene chloride. The combined methylene
chloride layers were washed with 100 ml of brine, and dried over
20 magnesium sulfate. The methylene chloride was removed with
aspirator vacuum at about 35°C, and the remaining dimethylformamide
was removed with vacuum at about 45°C. The crude material was
left on a vacuum line overnight at room temperature.

25 The crude material was then dissolved in 100 ml of methylene
chloride at room temperature. Triphenylphosphine (8.91 g, 34 mmol)

was added and dissolved with stirring. An argon purge was started and the mixture was cooled to 0°C with an ice bath. The N-bromosuccinimide (6.05 g, 34 mmol) was added portionwise over two minutes. The mixture was stirred for 1.5 hours at 0°C. The methylene chloride was removed with vacuum and gave a purple oil. This oil was triturated with 200 ml of ether with constant manual stirring. During this time the oil became very thick. The ether solution was decanted and the oil was triturated with 100 ml of ether. The ether solution was decanted and the oil was again triturated with a 100 ml portion of ether. The ether was decanted and the combined ether solution was allowed to stand for about two hours to allow the triphenylphosphine oxide to crystallize. The ether solution was decanted from the crystals and the solid was washed with 100 ml of ether. The volume of the combined ether extracts was reduced with vacuum until a volume of about 25 ml was obtained. This was allowed to stand overnight at 0°C. Ether (10 ml) was added to the cold mixture, which was mixed to suspend the solid. The mixture was percolated through a column of 45 g of silica gel and eluted with ether, and 75 ml fractions were collected. The fractions that contained product, as determined by thin layer chromatography, were pooled and the ether was removed with vacuum. This yielded 10.1 g of crude product. The material was flash chromatographed on silica gel with hexane, changing to 9:1 hexane:ether. The product-

containing fractions were pooled and the solvents removed with vacuum. This yielded 7.4 g (57% yield) of pure product.

A mixture of 10% palladium on carbon (1 g) and a solution of the benzyl ester (10 g) in 150 ml of methanol was hydrogenolyzed at
5 25 psi for two hours. The mixture was filtered over celite and the residue was washed with methanol. The solvent was evaporated to give a viscous oil in quantitative yield.

Reaction of the bromide with 1,1,2-trimethyl-[1H]-
10 benz[e]indole was carried out as described in Example 1.

EXAMPLE 6

Bis(ethylcarboxymethyldihydroxyl)indocyanine Dye (FIG. 3)

The hydroxy-indole compound is readily prepared by a known method (P. L. Southwick et al., One pot Fischer synthesis of
15 (2,3,3-trimethyl-3-H-indol-5-yl)-acetic acid derivatives as intermediates for fluorescent biolabels. *Org. Prep. Proced. Int. Briefs*, 1988, 20(3), 279-284). Reaction of p-carboxymethylphenylhydrazine hydrochloride (30 mmol, 1 equiv.) and 1,1-bis(hydroxymethyl)propanone (45 mmole, 1.5 equiv.) in acetic acid (50 mL) at room temperature for 30 minutes
20 and at reflux for one minute gives (3,3-dihydroxymethyl-2-methyl-3-H-indol-5-yl)-acetic acid as a solid residue. The reaction of 3-bromopropyl-N,N-bis(carboxymethyl)amine, which was prepared as described in Example 5, with the intermediate indole and subsequent

reaction of the indole intermediate with glutacanaldehyde dianil monohydrochloride (see Example 1) gives the desired product.

EXAMPLE 7

5 Synthesis of Bis(propylcarboxymethyl)indocyanine Dye (FIG. 4)

The intermediate 2-chloro-1-formyl-3-hydroxymethylenecyclohexane was prepared as described in the literature (G. A. Reynolds and K. H. Drexhage, Stable heptamethine pyrylium dyes that absorb in the infrared. *J. Org. Chem.*, 1977, 42(5), 885-888). Equal volumes (40 mL each) of dimethylformamide (DMF) and dichloromethane were mixed and the solution was cooled to -10°C in an acetone-dry ice bath. Under argon atmosphere, phosphorus oxychloride (40 mL) in dichloromethane was added dropwise to the cool DMF solution, followed by the addition of 10 g of cyclohexanone.

10 The resulting solution was allowed to warm to room temperature and was refluxed for six hours. After cooling to room temperature, the mixture was poured into ice-cold water and stored at 4°C for twelve hours. About 8 g of yellow powder was obtained after filtration.

15 Condensation of the cyclic dialdehyde with the indole intermediate is carried out as described in Example 1. Further functionalization of the dye with bis isopropylidene acetal protected monosaccharide was accomplished by the method described in the literature (J. H. Flanagan, et al., Near infrared heavy-atom-modified fluorescent dyes for base-

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calling in DNA-sequencing application using temporal discrimination.

Anal. Chem., 1998, 70(13), 2676-2684).

EXAMPLE 8

5 Synthesis of Bis(ethylcarboxymethyl)indocyanine Dye (FIG. 5)

These dyes are prepared as described in Example 7.

These dyes absorb in the infrared region. The typical example shown in FIG. 5 has an estimated absorption maximum at 1036 nm.

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EXAMPLE 9

Synthesis of Peptides

The procedure described below is for the synthesis of Octreotate. The amino acid sequence of Octreotate is: D-Phe-Cys'-Tyr-D-Trp-Lys-Thr-Cys'-Thr (SEQ ID NO:1), wherein Cys' indicates the
15 presence of an intramolecular disulfide bond between two cysteine amino acids. Other peptides of this invention were prepared by a similar procedure with slight modifications in some cases.

The octapeptide was prepared by an automated
fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis using a
20 commercial peptide synthesizer from Applied Biosystems (Model 432A SYNERGY Peptide Synthesizer). The first peptide cartridge contained Wang resin pre-loaded with Fmoc-Thr on 25 μ mole scale. Subsequent cartridges contained Fmoc-protected amino acids with side chain

protecting groups for the following amino acids: Cys(Acm), Thr(t-Bu), Lys(Boc), Trp(Boc) and Tyr(t-Bu). The amino acid cartridges were placed on the peptide synthesizer and the product was synthesized from the C- to the N-terminal position. The coupling reaction was

5 carried out with 75 μ moles of the protected amino acids in the presence of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/N-hydroxybenzotriazole (HOBt). The Fmoc protecting group was removed with 20% piperidine in dimethylformamide. After the synthesis was complete, the thiol group

10 was cyclized with thallium trifluoroacetate and the product was cleaved from the solid support with a cleavage mixture containing trifluoroacetic acid (85%):water (5%):phenol (5%):thioanisole (5%) for six hours. The peptide was precipitated with t-butyl methyl ether and lyophilized with water:acetonitrile (2:3) mixture. The peptide was

15 purified by HPLC and analyzed with LC/MS.

Octreotide, D-Phe-Cys'-Tyr-D-Trp-Lys-Thr-Cys'-Thr-OH (SEQ ID NO:2), wherein Cys' indicates the presence of an intramolecular disulfide bond between two cysteine amino acids, was prepared by the same procedure.

20 Bombesin analogs were prepared by the same procedure except that cyclization with thallium trifluoroacetate was not needed. Side-chain deprotection and cleavage from the resin was carried out with 50 μ L each of ethanedithiol, thioanisole and water, and 850 μ L of

trifluoroacetic acid. Two analogues were prepared: Gly-Ser-Gly-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂ (SEQ ID NO:3) and Gly-Asp-Gly-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂ (SEQ ID NO:4).

Cholecystokinin octapeptide analogs were prepared as described for Octreotate without the cyclization step. Three analogs were prepared: Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH₂ (SEQ ID NO:5); Asp-Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH₂ (SEQ ID NO:6); and D-Asp-Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH₂ (SEQ ID NO:7), where Nle is norleucine.

A neurotensin analog, D-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu (SEQ ID NO:8), was prepared as described for Octreotate without the cyclization step.

EXAMPLE 10

Synthesis of Peptide-Dye Conjugates (FIG. 6)

The method described below is for the synthesis of Octreotate-cyanine dye conjugates, but a similar procedure is used for the synthesis of other peptide-dye conjugates.

Octreotate was prepared as described in Example 9 but the peptide was not cleaved from the solid support and the N-terminal Fmoc group of Phe was retained. The thiol group was cyclized with thallium trifluoroacetate and the Phe was deprotected to liberate the free amine. Bisethylcarboxymethylindocyanine dye (53 mg, 75 μ moles) was added to an activation reagent consisting of a 0.2 M solution of

HBTU/HOBt in DMSO (375 μ L), and 0.2 M solution of diisopropylethylamine in DMSO (375 μ L). The activation was complete in about 30 minutes and the resin-bound peptide (25 μ moles) was added to the dye. The coupling reaction was carried out at room temperature for three hours. The mixture was filtered and the solid residue was washed with DMF, acetonitrile and THF. After drying the green residue, the peptide was cleaved from the resin and the side chain protecting groups were removed with a mixture of 85% trifluoroacetic acid, 2.5% water, 2.5% thioanisole and 2.5% phenol. The resin was filtered and cold t-butyl methyl ether (MTBE) was used to precipitate the dye-peptide conjugate, which was dissolved in an acetonitrile:water (2:3) mixture and lyophilized. The product was purified by HPLC to give the monoOctreotate-Bisethylcarboxymethylindocyanine dye (Cytate 1, 80%) and the bisOctreotate-Bisethylcarboxymethylindocyanine dye (Cytate 2, 20%). The monoOctreotate conjugate is obtained almost exclusively (>95%) over the bis conjugate by reducing the reaction time to two hours. However, this also leads to incomplete reaction, and the free Octreotate must be carefully separated from the dye conjugate in order to avoid saturation of the receptors by the non-dye conjugated peptide.

Octreotate-bispentylcarboxymethylindocyanine dye was prepared as described above with some modifications.

Bispentylcarboxymethylindocyanine dye (60 mg, 75 μ moles) was

added to an activation reagent consisting of a 0.2 M solution of HBTU/HOBt in DMSO (400 μ L), and a 0.2 M solution of diisopropylethylamine in DMSO (400 μ L). The activation was complete in about 30 minutes and the resin-bound peptide (25 μ moles) was added to the dye. The reaction was carried out at room temperature for three hours. The mixture was filtered and the solid residue was washed with DMF, acetonitrile and THF. After drying the green residue, the peptide was cleaved from the resin and the side chain protecting groups were removed with a mixture of 85% trifluoroacetic acid, 2.5% water, 2.5% thioanisole and 2.5% phenol. The resin was filtered and cold t-butyl methyl ether (MTBE) was used to precipitate the dye-peptide conjugate, which was dissolved in an acetonitrile:water (2:3) mixture and lyophilized. The product was purified by HPLC to give Octreotate-1,1,2-trimethyl-[1H]-benz[e]indole propanoic acid conjugate (10%), monoOctreotate-bis(pentylcarboxymethyl)indocyanine dye (Cytate 3, 60%) and bisOctreotate-bis(pentylcarboxymethyl)indocyanine dye (Cytate 4, 30%).

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EXAMPLE 11

Formulation of peptide-dye conjugates in dimethyl sulfoxide (DMSO)

The dye-peptide conjugates are sparingly soluble in water and require the addition of solubilizing agents or co-solvents. Addition

of 1-20% aqueous ethanol to the conjugates partially quenched the fluorescence intensity *in vitro* and the fluorescence was completely quenched *in vivo* (the conjugate was not detected by the charge coupled device (CCD) camera). Addition of 1-50% of DMSO either re-established or increased the fluorescence intensity of the conjugates *in vitro* and *in vivo*. The dye fluorescence remained intense for over one week. The DMSO formulations were well tolerated by experimental animals used for this invention.

EXAMPLE 12

Imaging of pancreatic ductal adenocarcinoma (DSL 6A) with Indocyanine Green (ICG)

5 A non-invasive *in vivo* fluorescence imaging apparatus was employed to assess the efficacy of contrast agents developed for tumor detection in animal models. A LaserMax Inc. laser diode of nominal wavelength 780 nm and nominal power of 40 mW was used. The detector was a Princeton Instruments model RTE/CCD-1317-K/2
10 CCD camera with a Rodenstock 10 mm F2 lens (stock #542.032.002.20) attached. An 830 nm interference lens (CVI Laser Corp., part # F10-830-4-2) was mounted in front of the CCD input lens such that only emitted fluorescent light from the contrast agent was imaged. Typically, an image of the animal was taken pre-injection
15 of contrast agent. This image was subsequently subtracted (pixel by pixel) from the post injection images. However, the background

subtraction was never done once the animal had been removed from the sample area and returned at a later time for images taken several hours post injection.

DSL 6A tumors were induced in male Lewis rats in the
5 left flank area by the introduction of material from a solid (donor) implant, and the tumors were palpable in approximately 14 days. The animals were anesthetized with xylazine: ketamine: acepromazine, 1.5: 1.5: 0.5 at 0.8 mL/kg via intramuscular injection. The area of the tumor (left flank) was shaved to expose the tumor and the surrounding
10 surface area. A 21 gauge butterfly equipped with a stopcock and two syringes containing heparinized saline was placed into the later tail vein of the rat. Patency of the vein was checked prior to administration of the ICG via the butterfly apparatus. Each animal received 500 μ L of a 0.42 mg/mL solution of ICG in water.

15 FIGS. 7A-B are tumor images at two minutes (FIG. 7A) and 30 minutes (FIG. 7B) post bolus injection of a 0.5 mL aqueous solution of ICG (5.4 μ m). Tetracarboxylic acid cyanine dyes were synthesized as shown in FIG. 2, with A = CH₂ or CH₂OCH₂; R₁ = R₂ = H (Formula 1) or R₁,R₂ = fused phenyl (Formula 2).

20 The Figures are false color images of fluorescent intensity measured at the indicated times, with images constrained to the tumor and a small surrounding area. As is shown, the dye intensity in the tumor is considerably diminished 30 minutes post-ICG injection.

EXAMPLE 13

Imaging of Prostatic Carcinoma (R3327-H) with Indocyanine Green (ICG)

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The imaging apparatus and the procedure used are described as in Example 12. Prostate tumors (Dunning R3327-H) were induced in young male Copenhagen rats in the left flank area from a solid implant. These tumors grow very slowly and palpable masses were present 4-5 months post implant. FIGS. 7C-D are images of a rat with an induced prostatic carcinoma tumor (R3327-H) imaged at two minutes (FIG. 7C) and 30 minutes (FIG. 7D) post injection.

The Figures are false color images of fluorescent intensity measured at the indicated times, with images constrained to the tumor and a small surrounding area. As is shown, the dye intensity in the tumor is considerably diminished 30 minutes post-ICG injection.

EXAMPLE 14

Imaging of Rat Pancreatic Acinar Carcinoma (CA20948) with Indocyanine Green (ICG)

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The imaging apparatus and the procedure used are described in Example 12. Rat pancreatic acinar carcinoma expressing the SST-2 receptor (CA20948) was induced by solid implant technique in the left flank area, and palpable masses were detected nine days post implant. The images obtained at 2 and 30 minutes post injection

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are shown in FIG. 7E-F. FIGS. 7E-F are images of a rat with an induced pancreatic acinar carcinoma (CA20948) expressing the SST-2 receptor imaged at two minutes (FIG. 7E) and 30 minutes (FIG. 7F) post injection.

5 The Figures are false color images of fluorescent intensity measured at the indicated times, with images constrained to the tumor and a small surrounding area. As is shown, the dye intensity in the tumor is considerably diminished and almost absent 30 minutes post-ICG injection.

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EXAMPLE 15

Imaging of Rat Pancreatic Acinar Carcinoma (CA20948) with Cytate 1

 The imaging apparatus and the procedure used are described in Example 12, except that each animal received 500 μ L of a
15 1.0 mg/mL solution of Cytate 1 solution of 25% dimethylsulfoxide in water.

 Rat pancreatic acinar carcinoma expressing the SST-2 receptor (CA20948) were induced by solid implant technique in the left flank area, and palpable masses were detected 24 days post
20 implant. Images were obtained at various times post injection. Uptake into the tumor was seen at two minutes but was not maximal until about five minutes.

FIGS. 8A-B show a comparison of the uptake of ICG and Cytate 1 at 45 minutes in rats with the CA20948 tumor cell line. By 45 minutes the ICG has mostly cleared (FIG. 8A) whereas the Cytate 1 is still intense (FIG. 8B). This dye fluorescence remained intense in the tumor for several hours post-injection.

EXAMPLE 16

Imaging of Rat Pancreatic Acinar Carcinoma (CA20948) with Cytate 1 Compared with Imaging with Indocyanine Green

Using indocyanine green (ICG), three different tumor lines were imaged optically using a CCD camera apparatus. Two of the lines, DSL 6/A (pancreatic) and Dunning R3327H (prostate) indicated slow perfusion of the agent over time into the tumor and reasonable images were obtained for each. The third line, CA20948 (pancreatic), indicated only a slight but transient perfusion that was absent after only 30 minutes post injection. This indicated no non-specific localization of ICG into this line compared to the other two tumor lines, suggesting a different vascular architecture for this type of tumor (see FIGS. 7A-F). The first two tumor lines (DSL 6/A and R3327H) are not as highly vascularized as CA20948, which is also rich in somatostatin (SST-2) receptors. Consequently, the detection and retention of a dye in this tumor model is a good index of receptor-mediated specificity.

Octreotate is known to target somatostatin (SST-2) receptors, hence, cyano-Octreotates (Cytate 1 and Cytate 2) were

prepared. Cytate 1 was evaluated in the CA20948 Lewis rat model. Using the CCD camera apparatus, strong localization of this dye was observed in the tumor at 90 minutes post injection (Figure 9A). At 19 hours post injection the animal was again imaged (Figure 9B). Tumor
5 visualization was easily observed showing specificity of this agent for the SST-2 receptors present in this tumor line. As a control, the organs were imaged again (Figure 10A) and the image was compared with that of the same tissues in the uninjected rat (Figure 10B).

Individual organs were removed and imaged. High uptake
10 of the material was observed in the pancreas, adrenals and tumor tissue, while heart, muscle, spleen and liver indicated significantly lower uptake (Figure 11). These data correlate well with radiolabeled Octreotate in the same model system (M. de Jong, et al. *Cancer Res.* 1998, 58, 437-441).

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EXAMPLE 17

Monitoring of the blood clearance profile of peptide-dye conjugates

A laser of appropriate wavelength for excitation of the dye chromophore was directed into one end of a fiber optic bundle and
20 the other end was positioned a few millimeters from the ear of a rat. A second fiber optic bundle was also positioned near the same ear to detect the emitted fluorescent light and the other end was directed into the optics and electronics for data collection. An interference filter

(IF) in the collection optics train was used to select emitted fluorescent light of the appropriate wavelength for the dye chromophore.

Sprague-Dawley or Fischer 344 rats were used in these studies. The animals were anesthetized with urethane administered via
5 intraperitoneal injection at a dose of 1.35 g/kg body weight. After the animals had achieved the desired plane of anesthesia, a 21 gauge butterfly with 12" tubing was placed in the lateral tail vein of each animal and flushed with heparinized saline. The animals were placed onto a heating pad and kept warm throughout the entire study. The
10 lobe of the left ear was affixed to a glass microscope slide to reduce movement and vibration.

Incident laser light delivered from the fiber optic was centered on the affixed ear. Data acquisition was then initiated, and a background reading of fluorescence was obtained prior to
15 administration of the test agent. For Cytates 1 or 2, the peptide-dye conjugate was administered to the animal through a bolus injection, typically 0.5 to 2.0 mL, in the lateral tail vein. This procedure was repeated with several dye-peptide conjugates in normal and tumor-bearing rats. Representative profiles as a method to monitor blood
20 clearance of the peptide-dye conjugate in normal and tumor-bearing animals are shown in FIGS. 12 to 16. The data were analyzed using a standard sigma plot software program for a one-compartment model.

In rats treated with Cytates 1 or 2, the fluorescence signal rapidly increased to a peak value. The signal then decayed as a function of time as the conjugate cleared from the bloodstream. FIG. 12 shows the clearance profile of Cytate 1 from the blood of a normal
5 rat monitored at 830 nm after excitation at 780 nm. FIG. 13 shows the clearance profile of Cytate 1 from the blood of a pancreatic tumor (CA20948)-bearing rat also monitored at 830 nm after excitation at 780 nm.

FIG. 14 shows the clearance profile of Cytate 2 from the
10 blood of a normal rat, and FIG. 15 shows the clearance profile of Cytate 2 from the blood of a pancreatic tumor (CA20948)-bearing rat, monitored at 830 nm after excitation at 780 nm.

FIG. 16 shows the clearance profile of Cytate 4 from the blood of a normal rat monitored at 830 nm after excitation at 780 nm.

15 It should be understood that the embodiments of the present invention shown and described in the specification are only specific embodiments of the inventors who are skilled in the art and are not limiting in any way. Therefore, various changes, modifications or alterations to those embodiments may be made or resorted to
20 without departing from the spirit of the invention and the scope of the following claims. The references cited are expressly incorporated by reference herein in their entirety.